REAL LIFE EXPERIENCES USING THE WRAIR WHOLE BLOOD AND PYRIDOSTIGMINE ASSAYS

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ABSTRACT

Walter Reed Army Institute of Research Whole Blood (WRAIR WB) cholinesterase assay rapidly determines the concentrations of both AChE and BChE in unprocessed whole blood, uses a minimally invasive blood sampling technique (finger prick), and is fully automated (using the Biomek 2000 robotic system). In human blood from volunteers given pyridostigmine bromide (30 mg single dose), RBC-AChE was maximally inhibited by about 30% after 2.5 h, with recovery to 95% after 24 h. After *ex vivo* addition of GD to inhibit AChE, and subsequent PB and GD removal, we found that all the protected (by PB pretreatment) AChE activity was recovered after 24h (essentially within 6 h). The same blood samples were evaluated using a WRAIR-developed HPLC method to directly quantify PB. The assay is sensitive to 1 $ng.mL^{-1}$ with high precision. There was excellent agreement between levels of PB in plasma and AChE inhibition observed. Thus, these assays provide validated support for PB pretreatment as protection against organophosphate chemical warfare agents.

INTRODUCTION

The concentration of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in blood is potentially a stable biomarker of suppressed and/or heightened central and peripheral nervous system activity. Exposure to nerve agents, organophosphates (OPs), pesticides, anesthetics, terrorists' chemical agents, cocaine, and some neurodegenerative disease states selectively reduces AChE or BChE activity. Therefore, blood cholinesterase activity can be exploited as a tool for confirming exposure to the agents and possible treatments. The current tests used to determine cholinesterase levels in blood, however, are not U.S Food and Drug Administration (FDA) approved, and have significant drawbacks including the lack of standardization, long turn-around times, and difficulty in comparing results between different laboratories. In part, this is because clinical determination of cholinesterase levels in blood typically

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utilize three different techniques: Michel, pH stat, or Ellman, and normally determine either RBC-AChE or serum cholinesterase (BChE) concentrations, but not usually both.[1-2] In addition, given the potential increase in urban terrorism that may include the use of chemical warfare organophosphate agents, Federal, State, and local authorities need a reliable, fast, inexpensive, and standard method for confirming such an assault in order to initiate appropriate containment, decontamination, and treatment measures.

Therapeutic drugs have a wide effect on cholinesterases[3]; minor inhibition is observed with a variety of anesthetics. High inhibition is observed (purposely) with pyridostigmine bromide, its therapeutic advantage being to increase the muscle strength in myasthenia gravis patients by inhibiting acetylcholinesterase that results in an accumulation of acetylcholine at cholinergic synapses[4]. Pesticides are also inhibitors of cholinesterases, and their toxicity is well documented[5]. Yet it is the organophosphate chemical warfare agents that are some of the most potent and irreversible inhibitors that can produce excessive accumulation of acetylcholine, and ultimately a cholinergic crisis in man leading to death[6]. Although the physiological state of an individual, drugs, pesticides, and chemical warfare agents affect cholinesterases, only for the latter two are routine cholinesterase measurements utilized.

We have developed and described[7] a new methodology - The Walter Reed Army Institute of Research Whole Blood (WRAIR WB) cholinesterase assay - that quickly determines the concentrations of AChE and BChE simultaneously in unprocessed, whole blood. We describe a robust protocol to quickly and simultaneously provide accurate and precise blood concentrations of both AChE and BChE. To accomplish this assay, we measure the activity of whole blood in the presence of three substrates for AChE and BChE, which provide redundancy and independent determination of both AChE and BChE activities. This is possible because: 1) each protein possesses a different affinity and sensitivity for each of its substrates and 2) a direct relationship exists between activity and enzyme concentration.

METHODS

1. Comparison of Cholinesterase Assays. As previously described[7], the final concentrations of the substrates for the 96-well microtiter plate in the WRAIR WB assay were 1 mM each of acetylthiocholine iodide (ATC), propionylthiocholine iodide (PTC), butyrylthiocholine iodide (BTC), and 0.2 mM 4,4'-dithidiopyridine (DTP), the indicator for the hydrolyzed thiocholine (UV absorbance at 324 nm). To perform the cholinesterase assays, fresh human whole blood was collected from subjects and stored in heparin Vacutainers® at 4°C (WRAIR human use protocol #776). (1) A small aliquot of blood, typically 10 µL, which was diluted 20-fold in distilled water, was placed in the well to give a final volume of 300 µL (50 mM sodium phosphate buffer, pH 8.0) containing the appropriate substrate and DTP, and performed in triplicate. Following a sixty second pre-read shaking to mix the contents thoroughly, a four-minute kinetic assay[8] was performed on each plate using a Molecular Devices SpectraMax Plus microtiter spectrophotometer (Sunnyvale, CA), interfaced to a Beckman-Coulter Biomek 2000 robotic station, that performed all the necessary sample handling steps. Each well was read at twelve second intervals, interspersed with three second shaking. The data were subjected to linear least squares analysis, from which the activities of AChE and BChE were calculated using SoftMax v4.3 and an Excel spreadsheet.

The WRAIR WB assay was compared to the following alternative cholinesterase assays, all of which include a significantly greater number of processing steps that are time consuming and labor intensive. (2) A sample of the same heparinized blood was analyzed for AChE and BChE content using the procedure of the Test-Mate OP™ system, following the manufacturer's guidelines. (3) To determine the cholinesterase activities using the COBAS/FARA clinical chemistry analyzer (Roche Diagnostics Corporation, Indianapolis, IN), the blood sample was centrifuged for 5 minutes in a fixed rotor microfuge at 14,000 rpm (9,800 x g). Plasma from each tube was carefully removed and utilized for BChE analysis.

The remaining RBCs in each of the 140 tubes were mixed and diluted 50-fold by placing 20 µL of RBCs into microtubes containing 980 µL of 1% Triton X-100 in saline. The plasma was diluted 15-fold by placing 68 µL plasma into microtubes containing 932 µL of 1% Triton X-100 in saline. (4) For the modified Michel method[9], centrifuged and resuspended RBCs were analyzed at the Cholinesterase Reference Laboratory (CRL) at the U.S. Army Center for Health Promotion and Preventive Medicine (CHPPM), APG, Edgewood, MD according to established procedures: (http://chppm-www.apgea.army.mil/dls/CHOLIN.HTM). (5) The microEllman assay uses the Ellman reagent, DTNB, in a microtiter plate[10]. Each blood sample was analyzed in triplicate for each specific cholinesterase assay; in other words, the test samples were analyzed 3 times for erythrocyte (AChE) and plasma (BChE) cholinesterase activity.

2. PB inhibition of RBC-AChE and protection assays. Aliquots of whole blood from twenty-four volunteers (nineteen given a single oral dose of pyridostigmine bromide (30 mg tablet), and five placebos) were withdrawn for the determination of RBC-AChE activity. A blood sample was taken before the PB dose (prescreen) and then at 2.5, 5, 8 and 24h after dosing. Blood samples were frozen at –80^{o}C and thawed immediately prior to the WRAIR WB cholinesterase assay.

To evaluate the protection afforded to RBC-acetylcholinesterase by PB, blood samples stored at –80^{o}C were thawed and then exposed to the irreversible organophosphate soman (GD, 1 µM) for 10 min at room temperature; these experiments were conducted at the USAMRICD, Aberdeen Proving Ground, MD). Free PB and GD were removed by using Biorad spin columns (6 cm) containing 300 mg of C_{18} (Waters, Milford, MA, #WAT010001). After adding 2.5µl of saponin (50 mg/ml) and vortexing for 1 min, 180 µL of the lysed whole blood, which could contain PB, GD, or other compounds, was added to the column and centrifuged at 1,000 x g for 2 min. This step removes free ligands from the blood, yet we are able to recover close to 100% of the AChE/BChE activity from the original blood sample. Although these samples were not exposed to oximes, we can remove interfering compounds including oximes such as 2-PAM and HI-6 (data not shown). Thus, after PB removal, we are able to monitor the time taken to achieve full return in activity (decarbamylation), and how much of the RBC-AChE is protected from GD exposure.

The % recovery of ChE activity is calculated as follows: % inhibited (PB or placebo samples) = 100 x (ChE U/mL of samples at times post-dosing)/(ChE U/mL pre-dosing). The % recovery (PB or placebo samples exposed to GD) = 100 x (ChE U/mL of GD samples at times post-dosing)/(ChE U/mL pre-dosing).

3. PB HPLC Assay. To verify compliance of ordered PB consumption (when expecting OP exposure), we have developed a sensitive HPLC technique to quantify PB in human blood as well as in Rhesus and rodent blood, and tissue. This is an alternate and confirmatory technique for measurement of PB levels directly to supplement the indirect assay by inhibition of ChEs. The PB HPLC technique is based on solid phase extraction, lyophilization for concentration, and HPLC of the reconstituted samples using strong-cation exchange chromatography and isocratic elution. The linear dynamic range of sensitivity covers at least 200 to 0.0025 ng of PB. This assay lends itself to high-throughput and direct PB quantification.

RESULTS

1. Comparison of Cholinesterase Assays. Currently, there is no mechanism for conversion of the AChE and BChE activities determined by one method to those of a different assay, for example the Michel pH method to the COBAS/FARA assays. To demonstrate a direct correlation between the WRAIR WB assay and these other assays, cholinesterase levels were measured in human blood samples by each assay in

parallel. The samples sizes were 112, 64, 112, and 64 for the Michel, COBAS/FARA, micro-Ellman, and TestMate OP kits respectively, with matching dilutions of the WRAIR WB cholinesterase method. To generate a wide dynamic range of human blood cholinesterase levels, the blood was inhibited with GD. Typically, one milliliter of a particular sample of human blood was aliquoted and mixed thoroughly with fourteen different concentrations of GD, ranging from 0 to 1 μM.

Figure 1 shows the WRAIR WB cholinesterase assay for AChE and BChE activities plotted in comparison to the other procedures: the Michel method (panels A and B) used by CHPPM; COBAS/FARA (panels C and D) from Roche Diagnostics Corporation; the micro-Ellman method (panel E); and the accepted field assay of the United States Army, the TestMate OP method (panel F). The figures clearly demonstrate that a co-linear relationship between the cholinesterases measured by the WWB method and the four alternate methods. Correlation coefficients for the fitted curves to the data (GraphPad Software, San Diego, CA) are shown by the R-value in each panel. The figures also demonstrate that results from established methods, e.g., the Michel assay, can be converted to those of the WWB ChE method by applying a simple transformation. This allows the extrapolation of cholinesterase databases constructed using prior methods to the values of the WWB ChE method.

2. PB protection assays. The three panels in figure 2 illustrate the inhibition of whole blood AChE in the nineteen volunteers who took PB. Maximal AChE inhibition is observed at 2.5 h post PB dosing, with return to pre-dosing levels achieved after 24 h. At 2.5h, nineteen PB-dosed individuals yielded a mean inhibition of AChE of 29.9%. The dashed lines in figure 2 represents the individuals receiving only the placebo and therefore little change in AChE activity is observed. PB-induced reversible AChE inhibition is variable, and could be due to a number of factors including individual PB absorption, weight, sex, age, or other variables including food intake. Nevertheless, as long as the blood samples were kept frozen at -80°C, the PB inhibition of AChE remained unchanged even when stored for 6 months post-dose (data not shown), indicating that the PB and PB-AChE complex is stable at this temperature.

The PB concentration in the same blood samples used to determine AChE inhibition was determined by our HPLC method. The panels in figure 3 shows that the maximal amount of PB in the blood was found at 2.5 h post PB dosing, identical to the greatest inhibition of AChE (figure 2). The placebo-treated human volunteers clearly showed no PB in the blood samples (dashed lines). Figure 4 demonstrates that a direct linear correlation ($r^2 = 0.98$) was established between the amount of PB measured in the blood (ordinate) and the inhibition of AChE (abscissa).

PB carbamylates or reversibly binds to AChE and thereby protects the enzyme from reaction with OPs. In particular, bound PB protects AChE from the rapidly aging GD, and resulting irreversible loss of AChE activity[11]. The PB-protected but inhibited AChE will be restored once the PB-AChE complex spontaneously decarbamylates, which occurs after GD is cleared from the blood. To demonstrate this, the PB-AChE blood with the highest inhibition (at 2.5 h post-dose, see figure 2) was exposed to GD (1 μM), and then rapidly centrifuged through a column to remove any free GD and PB. Under these circumstances, any RBC-AChE not protected by PB would be irreversibly inhibited by GD. In contrast, any RBC-AChE protected by PB would spontaneously decarbamylate over time, and this enzyme's activity would be restored. In figure 5, solid bars represent AChE without any PB (placebo), while crossed bars represent AChE from an individual receiving the 30 mg PB tablet. In the first step, after GD treatment, no AChE activity is observed by the WRAIR assay in either the placebo or PB samples. However, after column washing to remove free PB and GD, and a 24 h period to allow for complete decarbamylation, the PB-inhibited AChE is restored to the level that was initially inhibited by PB (29.9% inhibition by PB before the column vs. 33.7% returned AChE activity post-column). Figure 5 also shows that recovery of AChE activity is essentially complete after 6 h. This demonstrates that RBC-AChE is protected by PB from *ex vivo* GD addition.

CONCLUSIONS

A method capable of providing fast, precise, and accurate AChE and BChE measurements has been developed, and can be directly correlated for cholinesterase activity with other more classical measurement techniques. Unlike the conventional clinical tests, the WRAIR WB procedure provides a more detailed picture of the patient's cholinesterase levels (i.e., both AChE and BChE), produces results in less than five minutes, and is capable of high-throughput screening of whole blood by employing state of the art robotics. Furthermore, by coupling data from the WRAIR assay with a database conversion of the other ChE assays described in the Introduction based on figure 1, a medically predictive tool should result that can establish a military or civilian personnel's exposure to insecticides, nerve agents, medicinally administered drugs (such as PB), narcotics, and anesthetics.

The recommended dose of PB as a pretreatment for organophosphate exposure is (http://www.fda.gov/cder/drug/infopage/Pyridostigmine_Bromide/default.htm) 90 mg per day (3 x 30 mg at 8 h intervals). After a singe 30 mg dose to human volunteers, we have demonstrated that the maximal inhibition of RBC-AChE occurs at 2.5 h, and that this reversibly inhibited PB-AChE complex can spontaneously decarbamylate resulting in the loss of PB inhibition and the restoration of the original AChE activity. In addition, the PB-AChE complex was spared from the inhibition and irreversible aging by the GD, since *ex vivo* exposure of RBC-AChE from human volunteers receiving only one 30 mg tablet of PB to a large excess of GD, then subsequent removal of any unbound PB and GD, yielded full recovery of the originally protected enzyme. In contrast, there was no return of AChE activity after GD exposure *ex vivo* in the human blood of volunteers receiving the placebo. Using a highly sensitive HPLC method to quantify PB levels in plasma, we found excellent agreement between the levels of PB and the AChE inhibition observed using the whole blood assay.

These data demonstrate that pyridostigmine bromide is an effective drug for prophylaxis against the lethal effects of GD nerve agent poisoning. Given the extensive cumulative experience with the use of PB in patients with myasthenia gravis[12] and the significantly higher doses prescribed over many years (up to 1.5 g/day), PB is a safe drug when used as a pretreatment for OP poisoning.

REFERENCES

1. Silver, A. in The Biology of Cholinesterases, (1974) North Holland Publishing Company, Amsterdam, p58-67.
2. St. Omer, V.E.V., Rottinghaus, G.E. Biochemical determination of cholinesterase activity in biological fluids and tissues. In: Ballantyne, B., Marrs, T.C., editors, Clinical and Experimental Toxicology of Organophosphates and Carbamates, Oxford: Butterworth & Heinemann, (1992), p.15-27.
3. Jann, M.W., Shirley, K.L. and Small, G.W. Clinical Pharmacokinetics and pharmacodynamics of cholinesterase inhibitors. Clin. Pharmacokinet. (2002), 41(10): 719-739.
4. Kaur, J. and Zhang, M. O. Molecular modeling and QSAR of reversible acetylcholinesterase inhibitors. Curr Med Chem. (2000) 7:273-294.
5. Golden, N. H. and Rattner, B. A. Ranking terrestrial vertebrate species for utility in biomonitoring and vulnerability to environmental contaminants. Rev. Environ. Contam. Toxicol. (2003) 176:67-136.
6. Taylor, P, in Goodman & Gilman's The Pharmacological Basis of Therapeutics. (8th edition), Gilman A.G., Rall T.W., Neis A.S., & Taylor P. (eds), (1990), p131-149.

7. Feaster, S.R., Gordon, R.K., Clark, C.C., Maxwell, D.M., Lenz, D.E., and Doctor, B.P. "Unprocessed Whole Blood Cholinesterase Levels: WRAIR Protocol Development and Validation" USAMRMC Bioscience Review (2000), Hunt Valley, MD.
8. Andersson, A., Isaksson, A., Brattstom, L. and Hultberg, B. Homocysteine and other thiols determined in plasma by HPLC and thiol-specific post column derivatization. Clin. Chem. (1993), 39: 1590-1597.
9. Ellin, R.I., Burkhardt, B.H. and Hart, R.D. A time-modified method for measuring red blood cell cholinesterase activity. Arch. Environ. Health (1973), 27: 48-49.
10. Doctor, B.P., Toker, L., Roth, E. and Silman, I. Microtiter assay for acetylcholinesterase. Anal. Biochem. (1987), 166:399-403.
11. Sidell, F.R. Response to a chemical warfare attack. Postgrad.Med. (1990), 88:70-84.
12. Juhn, M.S. Myasthenia gravis. Diagnostic methods and control measures for a chronic disease. Postgrad Med. (1993), 94(5):161-171.

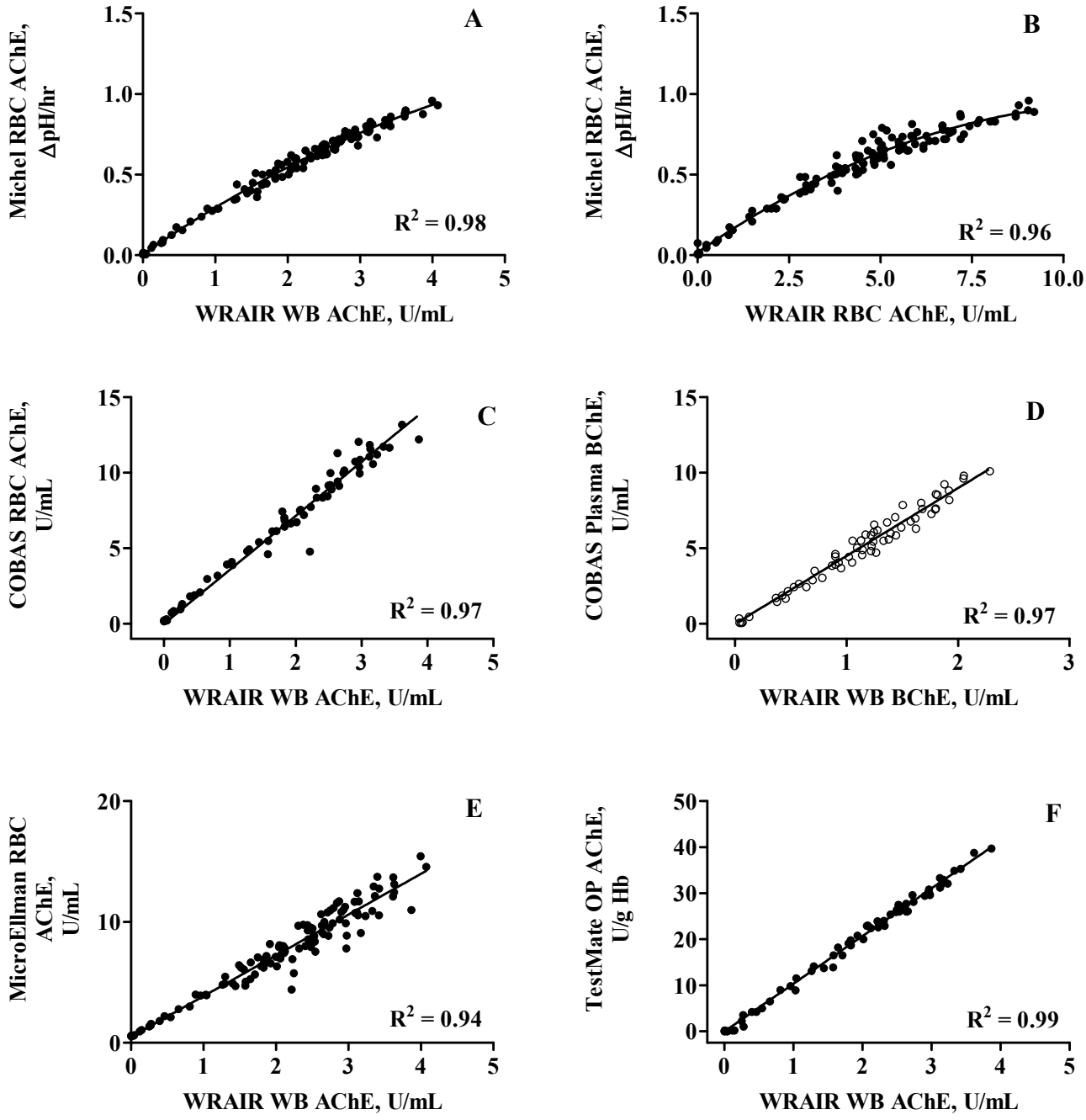

Figure 1. Closed circles, AChE; open circles, BChE. The WRAIR whole blood or RBC assay and Michel (A, B), COBAS/FARA (C, D), microEllman (E), and TestMate (F) exhibit high correlation coefficients (>0.94) for cholinesterase activity.

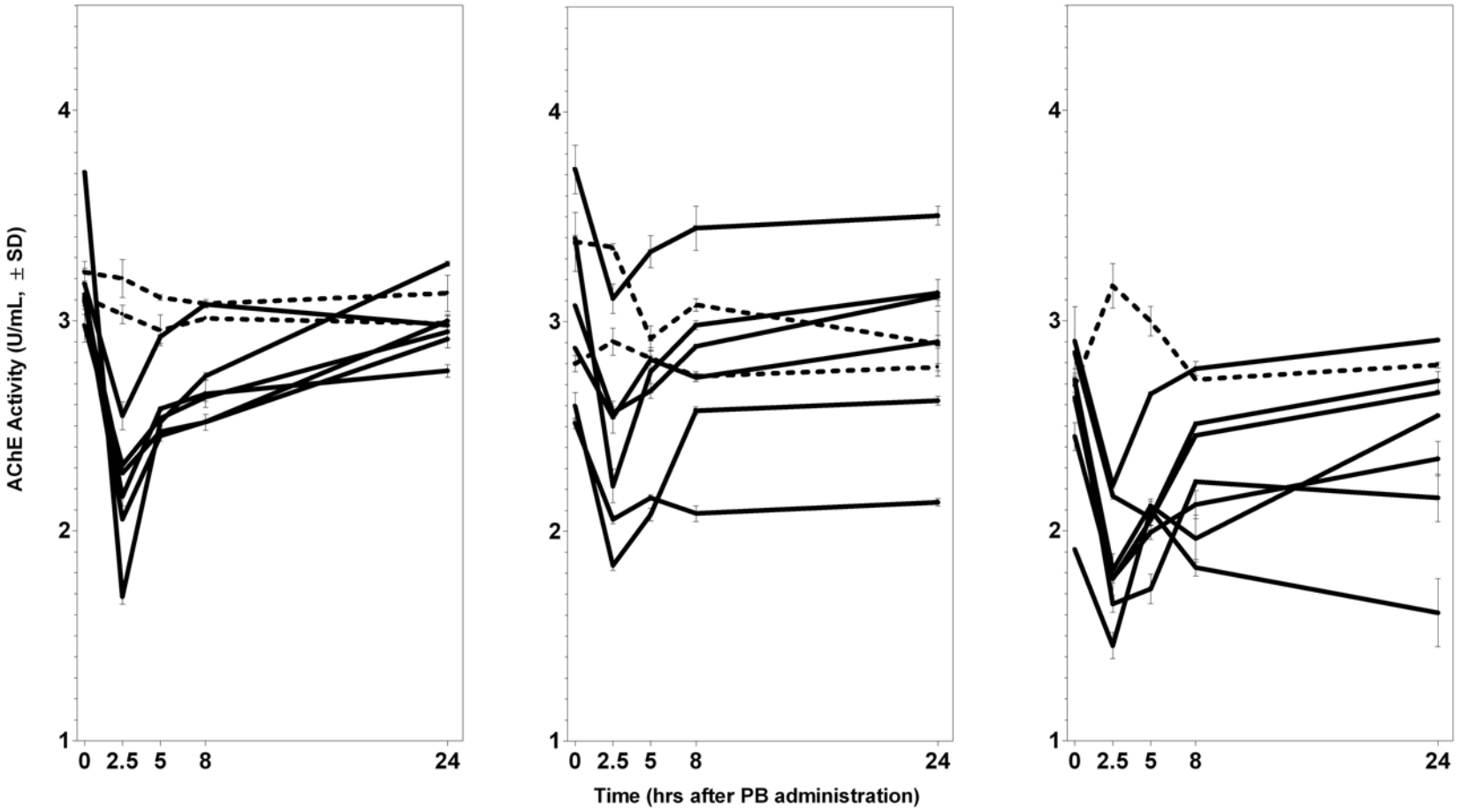

Figure 2. AChE activity in 19 human volunteers receiving a 30 mg PB tablet (solid lines) or the 5 placebos (dashed lines) as a function of time. Blood was drawn immediately before the PB dose (0 time, prescreen), and then at 2.5, 5, 8, and 24 h post-dose. Inhibition was maximal 2.5 h after PB administration for AChE. Results are displayed in three panels for clarity.

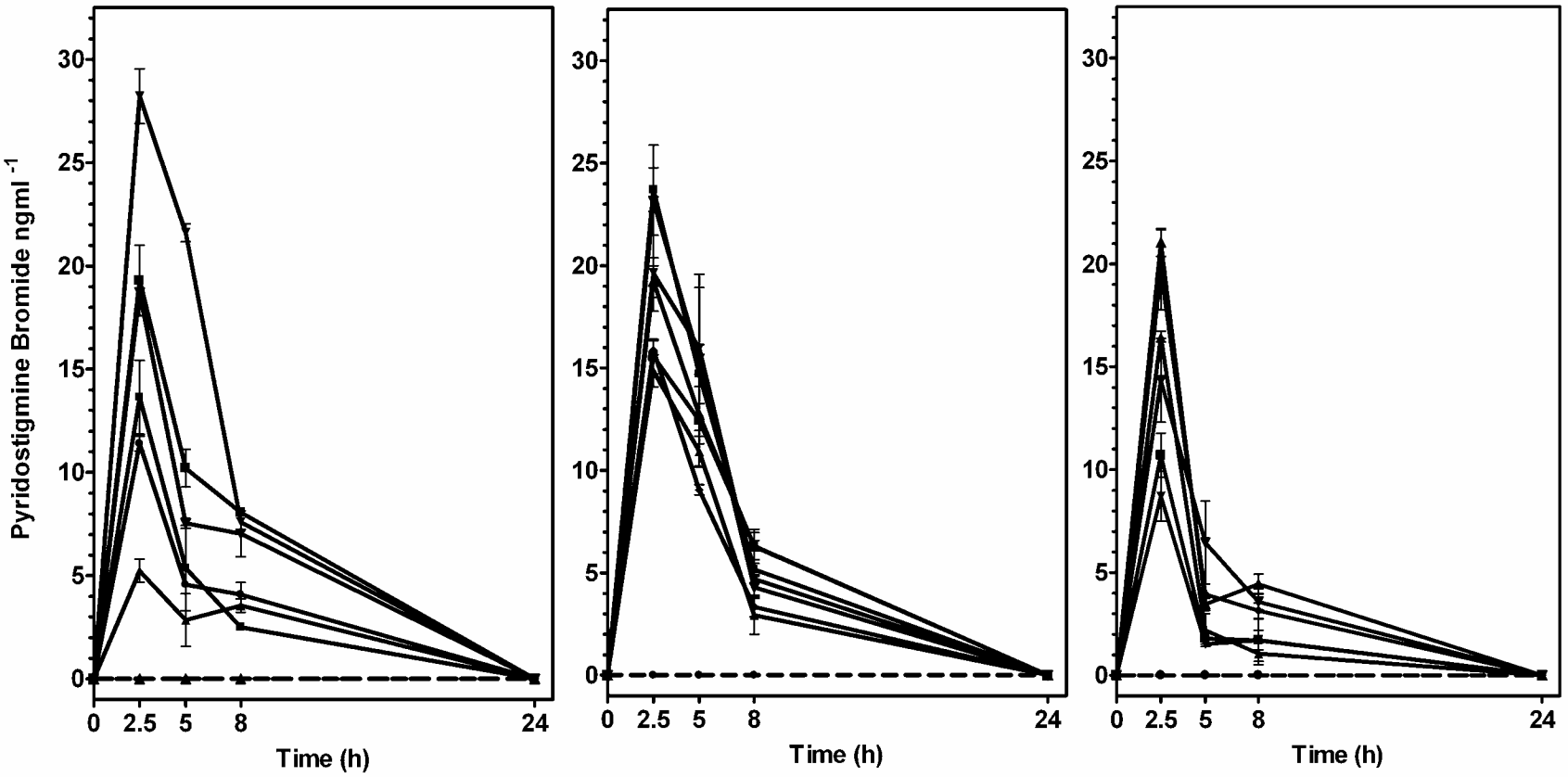

Figure 3. PB concentrations in the blood of 19 human volunteers receiving a 30 mg PB tablet (solid lines) and 5 individuals receiving placebo (dashed lines) as a function of time after PB dose. Results are displayed in three panels for clarity.

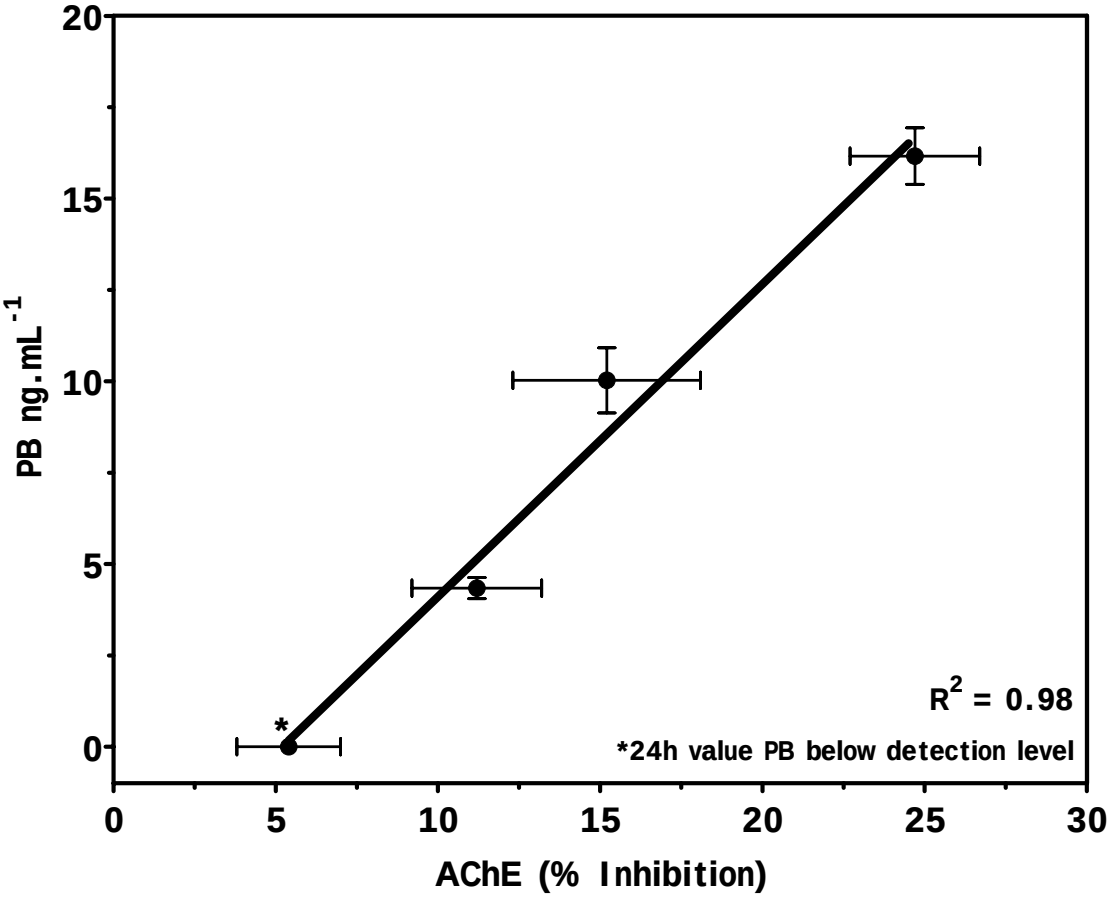

Figure 4. A direct linear correlation was established between the amount of PB measured in the blood by HPLC (ordinate) and the inhibition of AChE (abscissa). At 24 h, the PB level is below the detectable level.

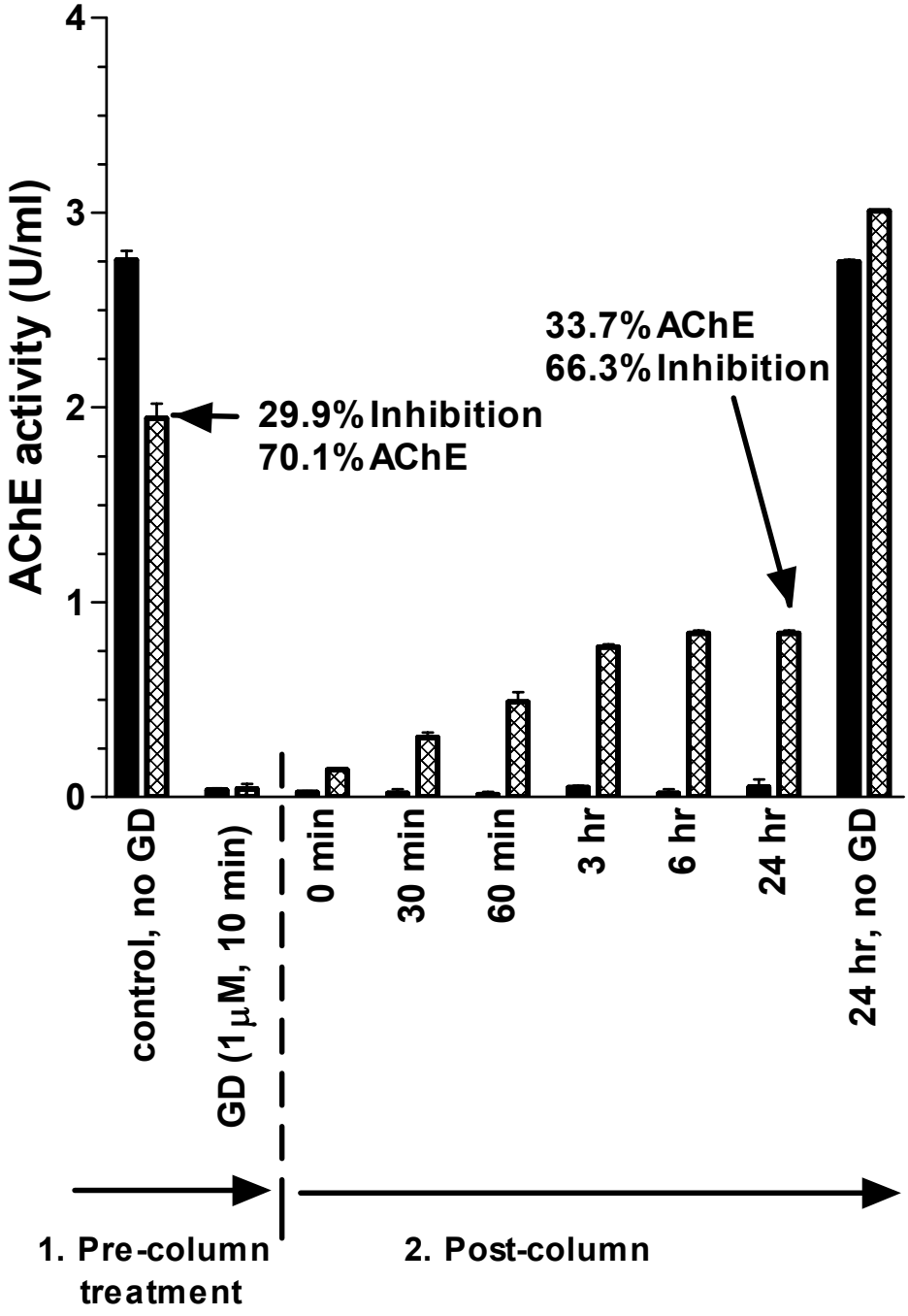

Figure 5. Effects of GD on AChE activity in whole blood from human volunteers who had taken pyridostigmine bromide (30 mg tablet). Blood was drawn 2.5 hrs post-dose when RBC-AChE is maximally inhibited by PB (left of dashed line). After GD exposure, PB and GD were removed using a C_{18} chromatography spin column (post-column treatment). The PB- protected AChE activity returned by 6 hr post-column.